High turnover of ezrin T567 phosphorylation: conformation, activity, and cellular function

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Zhu L, Zhou R, Mettler S, Wu T, Abbas A, Delaney J, Forte JG. High turnover of ezrin T567 phosphorylation: conformation, activity, and cellular function. Am J Physiol Cell Physiol 293: C874–C884, 2007. First published June 6, 2007; doi:10.1152/ajpcell.00111.2007.—In its dormant state, the membrane cytoskeletal linker protein ezrin takes on a NH2 terminal-to-COOH terminal (N-C) binding conformation. In vitro evidence suggests that eliminating the N-C binding conformation by Thr567 phosphorylation leads to ezrin activation. Here, we found for resting gastric parietal cells that the levels of ezrin phosphorylation on Thr567 are low and can be increased to a small extent (~40%) by stimulating secretion via the cAMP pathway. Treatment of cells with protein phosphatase can be increased to a small extent (~40%) by stimulating secretion via the cAMP pathway. Treatment of cells with protein phosphatase inhibitors led to a rapid, dramatic increase in Thr567 phosphorylation by 400% over resting levels, prompting the hypothesis that ezrin activity is regulated by turnover of phosphorylation on Thr567. In vitro and in vivo fluorescence resonance energy transfer analysis demonstrated that Thr567 phosphorylation opens the N-C interaction. However, even in the closed conformation, ezrin localizes to membranes by an exposed NH2 terminal binding site. Importantly, the opened phosphorylated form of ezrin more readily cosediments with F-actin and binds more tightly to membrane than the closed forms. Furthermore, fluorescence recovery after photobleaching analysis in live cells showed that the Thr567Asp mutant had longer recovery times than the wild type or the Thr567Ala mutant, indicating the Thr567-phosphorylated form of ezrin is tightly associated with F-actin and the membrane, restricting normal activity. These data demonstrate and emphasize the functional importance of reversible phosphorylation of ezrin on F-actin binding. A novel model is proposed whereby ezrin and closely associated kinase and phosphatase proteins represent a motor complex to maintain a dynamic relationship between the varying membrane surface area and filamentous actin length.

ezrin/radixin/moesin protein; motor complex; gastric parietal cell; fluorescence resonance energy transfer; fluorescence recovery after photobleaching

EZRIN, a cytoskeleton organizing protein, is a member of the ezrin/radixin/moesin (ERM) family (4, 11). ERM proteins are highly homologous in their primary structures and functions: they all have two ERM association domains (ERMADs); the NH2terminal (N)-ERMAD is a membrane binding domain, whereas the COOH terminal (C)-ERMAD is an F-actin binding domain (1, 30). In addition, the N-ERMAD and C-ERMAD bind to each other in intramolecular or intermolecular association and thus prevent the binding of ERM proteins to other molecules when ERM proteins are restrained to the dormant state (10, 39). By connecting F-actin and membrane, ezrin helps to form a platform for many molecules to meet, thus becoming directly involved in the regulation of cell shape, cell polarization, and signal transduction (4, 5, 24, 29).

Ezrin phosphorylation is often associated with the stimulation of cellular functions in a variety of cell models. In epidermoid carcinoma A-431 cells stimulated with EGF, increased phosphorylation on tyrosine, serine, and threonine was observed (3, 13). Phosphorylation sites Tyr145 and Tyr353 were later identified (21). In primary endothelial cells, TNF-α leads to ezrin phosphorylation and activates its function as a transcriptional repressor (19). Upon stimulation via the cAMP pathway, gastric parietal cells undergo dramatic morphological and functional changes that involve the indispensable role of ezrin within highly plastic apical microvillar structures (14, 28, 35). Characterization of the phosphorylation of ezrin from secretagogue-stimulated parietal cells revealed primary incorporation of 32P into ezrin (31, 37).

Phosphorylation at T567 in ezrin (the homologous sites are T564 in radixin and T558 in moesin) has received a great deal of attention because this phosphorylation event is believed to open up the NH2 terminal-to-COOH terminal (N-C) binding of ezrin, transforming ezrin into an active state with accessible domains for binding to membrane and F-actin. In vitro experiments with platelet moesin have indicated that T558 phosphorylation is necessary for binding to F-actin (16, 23). The increased affinity to F-actin is not likely due to the phosphate group itself, because COOH terminal radixin fragments bind to F-actin with similar efficiencies, whether phosphorylated on T564 or not (22). The same authors also found that T564 phosphorylation on the radixin COOH terminal suppressed its interaction with the NH2 terminal of radixin, in vitro evidence for abrogation of N-C binding by T564 phosphorylation. With cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged full-length radixin or radixin fragments, similar results were obtained with in vitro fluorescence resonance energy transfer (FRET) analysis (17). A study on the crystal structure of moesin revealed that phosphorylation at T558 weakened ERM N-C binding due to both electrostatic and steric effects (26).

It is well documented that ezrin T567 phosphorylation is associated with significant functional changes. The permeability of endothelial cells increased in response to TNF-α, which caused an increase of phosphorylation on conserved ezrin T567 (20). In an intestinal cell line, Akt2-dependent ezrin T567 phosphorylation led to Na+/H+ exchanger 3 translocation and activation (27). Using T558A and T558D mutation constructs, it was shown that moesin T558 phosphorylation via the Rho pathway (perhaps Rho kinase) plays a crucial role in the...
formation of microvilli (25). In LLC-PK1 epithelial cells stably transfected with these mutation constructs, the ezrin T567D (TD) mutant induced the formation of lamellipodia, membrane ruffles, and tufts of microvilli (12). The induction of microvilli by the ezrin TD mutant has also been reported in the early mouse embryo (8). Since these surface structures are required for cell movement, it is not a surprise that ezrin T567 phosphorylation is a key step in androgen-induced cell invasion (7).

The consequence of introducing the ezrin TD mutant into parietal cells was a surprise (38). This construct did not help in organizing and maintaining apical microvilli; instead, the apical membrane diminished, and H^+ -K^+ -ATPase, the major apical membrane protein responsible for proton pumping, was redirected to the basolateral surface with increasing levels of TD expression. Since T567 phosphorylation has not been previously detected in parietal cells, we originally proposed that it was not involved in the functional activation of ezrin in the parietal cell. However, using an antibody against T567-phosphorylated ezrin, we are now able to detect T567-phosphorylated endogenous ezrin in parietal cells. Thus, it is of great interest to reconsider ezrin T567 phosphorylation in parietal cells. Here, we characterized the phosphorylation of ezrin on T567 in vitro and in vivo. A model is proposed featuring the high turn over of ezrin T567 phosphorylation within a motor complex to maintain a dynamic relationship between the plasma membrane and supporting cytoskeleton.

MATERIALS AND METHODS

Reagents. The monoclonal anti-ezrin (AAS) antibody used in this study was purchased from Covance (Berkeley, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, and rhodamine-conjugated goat anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-labeled phalloidin was from Sigma. Phosphorylated ezrin (Thr(567)/radixin (Thr 564)/moesin (Thr 558) rabbit antibody (anti-T567P) was purchased from Cell Signaling Technology (Danvers, MA). This antibody has been widely used (12, 22) and tested rigorously in our laboratory to confirm its specificity to the T567 site and phosphorylation (data not shown). Rabbit antibody against green fluorescent protein (GFP) was purchased from Immunology Consultants Laboratory (Newberg, OR). Phosphatase inhibitors calyculin A (CLA; BioMol, Plymouth Meeting, PA) and okadaic acid (OKA; Sigma) were dissolved in DMSO to make 100 μM stock solutions. Working concentrations of CLA and OKA were 1 and 10 μM, respectively. When CLA and OKA were used, samples not treated with any of these drugs were mock treated with DMSO.

Recombinant adenoviruses. Recombinant adenovirus expressing YFP-ezrin-CFP [YEC-wild type (YEC-WT)] was as previously described (39). To make the constructs expressing the T567A (TA) and T567D (TD) mutants, the DNA sequence encoding the WT ezrin COOH terminal in plasmid DC311/YEC was replaced with the DNA sequence carrying the TA or TD mutation, which were prepared by double digestion of pDC311/EzT567A-CFP and pDC311/EzT567D-CFP (38) with restriction enzymes SmaI and NcoI. The resultant plasmids pDC311/YEC-T567A and pDC311/YEC-T567D were used to generate recombinant adenoviruses rAd/YEC-TA and rAd/YEC-TD using the AdMax system (Microbix Biosystems) as previously described (39).

Isolation of rabbit gastric glands and parietal cells. All procedures and treatments for handling animals were reviewed and approved by the Berkeley Animal Care and Use Committee. Gastric glands and parietal cells were isolated from New Zealand White rabbits (Oryctolagus cuniculus) as previously described (38). Glands were used in MEM suspension or cultured as isolated parietal cells as follows. For virus infection, cells (or glands) were plated onto Matrigel (Collaborative Biomedical, Stoney Brook, NY)-coated coverslips or dishes as described by Chew (6) and incubated at 37°C in culture medium A, which consisted of DMEM-F-12 (GIBCO-BRL), 20 mM HEPES, 0.2% BSA, 10 mM glucose, 8 mM EGTA, 1 μM site medium (which contained selenite, insulin, transferrin, and ethanolamine; S9290, Sigma), 1 mM glutamine, 100 U/ml penicillin-streptomycin, and 400 μg/ml gentamicin sulfate (pH 7.4). After 5 h of culture, the infection of cells or glands with adenoviral constructs was initiated.

Immunoblot analysis. Protein samples were separated by SDS-PAGE before being transferred onto nitrocellulose membranes. Membranes were blocked with 2% BSA in Tris-buffered saline [10 mM Tris (pH 7.0) and 150 mM NaCl] containing 0.05% Tween 20. Membranes were then probed with primary and secondary (HRP conjugated) antibodies. Results were then recorded by X-ray films with Western Lightning chemiluminescence substrate (Perkin-Elmer Life Sciences, Boston, MA). When reprobing was required, the blot was stripped in 2% SDS, 1% 2-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8) at 50°C for 30 min. The blot was then blocked and reprobed with another primary antibody. Signals from anti-T567P and anti-ezrin were found not to be carried to subsequent Western blot probedings.

Immunofluorescence microscopy. Parietal cells isolated from rabbit gastric glands were plated onto Matrigel-coated coverslips. After an infection with recombinant adenoviruses expressing fluorescence protein-tagged ezrin for 48 h, glands were fixed by 3.7% formaldehyde and permeabilized with 0.1% Triton X-100 before an incubation with rabbit anti-GFP. Afterward, cells were incubated with rhodamine-conjugated anti-rabbit antibody together with FITC-phalloidin. Images of rhodamine (excitation with a 543-nm laser and emission from 590–655 nm) and FITC (excitation with a 488-nm laser and emission from 505–558 nm) together with phase images were collected with a Zeiss LSM 510 META confocal microscope.

Analysis of acid secretion by gastric glands using [14C]aminopyrine uptake. The aminopyrine (AP) uptake assay measures the accumulation of AP in acidic spaces caused by the proton-pumping enzyme H^+ -K^+ -ATPase. A detailed description of this assay can be found in Ref. 38. When cimetidine, histamine, and 3-isobutyl-1-methyl xanthine (IBMX) were used, the final concentrations of these drugs were 100, 100, and 50 μM, respectively.

FRET measurement using spectrofluorometry. FRET measurements with a spectrofluorometer were performed as previously described (39) with slight modifications. Briefly, glands expressing YEC-WT, YEC-TA, or YEC-TD were homogenized in PBS containing 2 mM EDTA. The lysate was cleared by centrifugation at 300,000 g for 1 h at 4°C. Two emission spectra were recorded with the Spex spectrofluorometer: emission from 450 to 550 nm at an excitation of 425 nm and emission from 500 to 550 nm at an excitation of 485 nm. Emission spectra were corrected for background/autofluorescence using lysate of noninfected glands.

FRET measurement using confocal microscopy. Slight modifications were made to the confocal microscopy FRET method previously described (39). Briefly, water-immersion objectives were used with the Zeiss LSM 510 META microscope to record images of cultured cells. CFP images were collected using a 462- to 503-nm emission filter and an excitation laser of 458 nm; YFP images were collected using a 526- to 537-nm emission filter and an excitation laser of 514 nm; and FRET images were collected using a 526- to 537-nm emission filter and an excitation laser of 458 nm. Laser strength, pinhole, detector gain, amplifier gain, and scanning speed were optimized and fixed for all samples. Quantitation of FRET was performed with whole individual cells. The norms of the percentages of CFP and YFP bleed through under the microscope settings specified above were determined to be 0.15 (a; CFP) and 0.16 (b; YFP) using cells expressing pure CFP and pure YFP, respectively. No bleed through signal from CFP under the YFP filter setting was observed, and vice versa. Net FRET (nF) was calculated as nF = I_{FRET} - (I_{CFP} × a) - (I_{YFP} × b).
described in MATERIALS AND METHODS. Mean values of the AP ratio (2 samples for each treatment) were plotted with error bars representing the range. Glands treated in a similar way were taken for immunoblot assay for ezrin T567P (B) and for total ezrin (C). An increase of ezrin T567 phosphorylation was detected for the stimulated samples. Data shown here are representative of 5 independent gland preparations.

\[ \text{AP ratio} = \frac{\text{[H]}_{\text{CFP}} \times \text{I}_{\text{CFP}}}{\text{[H]}_{\text{YFP}} \times \text{I}_{\text{YFP}}} \]

where \( I_{\text{CFP}}, I_{\text{YFP}}, \) and \( I_{\text{FRET}} \) are the respective intensities of CFP, YFP, and FRET signals in each cell; and \( a \) and \( b \) are the corrections for CFP and YFP bleed through, respectively. The net FRET signal was normalized on the basis of relative CFP and YFP intensities according to the method described by Xia and Liu (34). Thus, normalized FRET \( (N_{\text{FRET}}) \) was calculated as \[ N_{\text{FRET}} = \frac{\text{CFP}}{\text{YFP}} \times I_{\text{CFP}} / I_{\text{YFP}} \times \frac{1}{2} \).

F-actin cosedimentation assay. Nonmuscle G-actin (Cytoskeleton) was reconstituted to 1 mg/ml in buffer A (0.2 mM CaCl₂, 0.2 mM ATP, 0.2 mM DTT, 0.02% NaN₃, and 5 mM Tris; pH 8.0) and centrifuged for 1 h at 300,000 g at 4°C before being used. Isolated rabbit gastric glands grown on 10-cm dishes were infected by recombinant adenoviruses expressing YEC-WT, YEC-TA, or YEC-TD. Two days after infection, glands were homogenized in buffer A. Lysates were cleared for 1 h at 300,000 g at 4°C before being used. Sedimentation was started by mixing 120 μl of actin solution, 150 μl of lysate, and 30 μl of 10× salt solution (20 mM MgCl₂ and 1 M KCl). Actin-only control and lysate-only controls were made simultaneously. Mixtures were incubated at room temperature for 1 h. F-actin and its binding proteins were pelletted by centrifugation for 1 h at 300,000 g at 4°C. All the cosedimentation samples and control samples were separated on SDS-PAGE gels. Gels were subjected to Coomassie blue staining and immunoblot assays with anti-GFP and anti-ezrin antibodies, respectively.

Membrane binding assay by sequential extraction. Isolated rabbit gastric glands grown on 10-cm dishes were infected by recombinant adenoviruses expressing YEC-WT, YEC-TA, or YEC-TD. Two days after infection, glands were sequentially extracted with low-salt buffer (buffer A, for cytosolic YEC), high-salt buffer (buffer C, for YEC weakly associated with membrane), and 1% Triton X-100 in buffer C (for YEC tightly associated with the membrane). During each extraction, gland pellets were homogenized for 50 strokes and centrifuged at 4°C at 300,000 g for 1 h. Extractions (supernatants) were collected and frozen at −80°C until further use, and pellets were subjected to the next extraction. For all samples, fluorescence (YFP) was measured at 526 nm with 485-nm excitation; this served as a comparator of expression. Extractions made from mock-infected glands were used to subtract background/antifluorescence from each sample. Percentages of the YEC distribution among all extractions were calculated based on the YFP intensity and size of each sample. Equal fractions of each extraction from all samples were separated by SDS-PAGE and analyzed by immunoblot analysis with anti-GFP, anti-ezrin, and anti-actin.

Fluorescence recovery after photobleaching analysis. Fluorescence recovery after photobleaching (FRAP) analysis was performed with a Zeiss LSM 510 META confocal microscope following the method described by van Drogen and Peter (33) with slight modifications. Gastric parietal cells grown in Matrigel-coated dishes were infected with recombinant adenoviruses expressing fluorescent protein-labeled ezrin, its TA mutant, or its TD mutant. Single or multiple interested areas from one cell were bleached with a 458-nm laser before three images of the whole cell were recorded. A time series of images were collected after photobleaching to allow for the fluorescence recovery of the bleached area. Images were taken using a 473- to 516-nm emission filter and an excitation laser of 458 nm. To compensate for the fluorescence loss during multiple scanning, the fluorescence intensities of the bleached area were normalized with the fluorescence intensity of an area far away from the bleached area (in most cases, an area from an unbleached cell). The intensity of the bleached area at half-recovery \( (t_{1/2}) \) was calculated as \[ I_{1/2} = I_0 + (I_0 - I_b)/2, \] where \( I_0 \) is the fluorescence intensity after photobleaching (intensity at \( t \) time) and \( I_b \) is the intensity at 100% recovery. With \( I_{1/2} \), the half-recovery time \( (t_{1/2}) \) was then calculated from the intensity vs. time trend line.

RESULTS

Phosphorylation of ezrin T567 is elevated upon cAMP-mediated stimulation of parietal cells. Gastric glands isolated from rabbits were treated with cimetidine or with histamine plus IBMX to provide resting and stimulated preparations, respectively. Radioactive \([^{14}C]\)AP was added to the preparations to monitor the acid secretory activity of parietal cells. Under conditions where there was a significant increase of acid secretion by the stimulated samples (Fig. 1A, compared with resting ones), glands were lysed for immunoblot analysis with

Fig. 1. Increased levels of ezrin T567 phosphorylation upon stimulation of gastric glands. Rabbit gastric glands were prepared and maintained in a resting state with cimetidine (CIM) or stimulated with histamine plus 3-isobutyl-1-methyl xanthine (H/I): A: \([^{14}C]\)aminopyridine (AP) uptake assay. Acid secretion by gastric glands was indirectly evaluated by AP accumulation as described in MATERIALS AND METHODS. Mean values of the AP ratio (2 samples for each treatment) were plotted with error bars representing the range. Glands treated in a similar way were taken for immunoblot assay for ezrin T567P (B) and for total ezrin (C). An increase of ezrin T567 phosphorylation was detected for the stimulated samples. Data shown here are representative of 5 independent gland preparations.

\[ \text{AP ratio} = \frac{\text{[H]}_{\text{CFP}} \times \text{I}_{\text{CFP}}}{\text{[H]}_{\text{YFP}} \times \text{I}_{\text{YFP}}} \]

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anti-T567P antibody (Fig. 1B). Two protein bands were detected from each sample: the top major band is T567-phosphorylated ezrin, as judged from molecular size and the fact that it overlays with the anti-ezrin signal; the bottom minor band is likely phosphorylated moesin. The data shown in Fig. 1 indicated that a small but significant increase of T567 phosphorylation occurred upon stimulation of gastric glands with histamine plus IBMX. From five independent experiments, an average increase of 43.3 ± 6.7% (mean ± SE, \( P < 0.05 \)) in the level of T567 phosphorylation was observed in stimulated glands.

**High turnover of T567 phosphorylation.** The detection of T567-phosphorylated ezrin and its increase with stimulation represents an apparent inconsistency with our earlier report (38), where expression of the ezrin TD mutant, mimicking constant phosphorylation, exerted a negative effect on acid secretion. To explain this inconsistency, we hypothesized that the function of ezrin depends on the high turnover of T567 phosphorylation, which keeps the total phosphorylation level low. This hypothesis was tested by treating gastric glands with CLA or OKA, both being specific inhibitors for the protein phosphatase 1 and 2A families (18). Compared with resting glands, treatment with CLA alone was able to stimulate acid secretion (Fig. 2A), similar to results reported previously (32). Treatment with OKA also produced a slight stimulation of acid secretion; differences in response to the inhibitors suggests that the substrates of CLA are different from those of OKA. These samples were then analyzed by immunoblot analysis with anti-T567P (Fig. 2B). Again, there was a slight increase of ezrin T567 phosphorylation when glands were stimulated via the cAMP-mediated pathway (i.e., histamine plus IBMX). The

![Fig. 3. Localization of yellow fluorescent protein (YFP)-ezrin-cyan fluorescent protein (CFP) constructs (YEC constructs). Rabbit gastric parietal cell cultures were infected for 48 h with recombinant adenoviruses (rAd) expressing YEC constructs as wild-type ezrin (YEC-WT), its T567A mutant (YEC-TA), or its T567D mutant (YEC-TD). Cells were fixed and permeabilized before incubation with rabbit antibody against fluorescent protein. After being washed, cells were incubated with rhodamine anti-rabbit IgG and FITC-phalloidin. Thus, the collected green images represent F-actin and the collected red images represent the indicated YEC constructs. Bars = 10 \( \mu \text{m} \).](http://ajpcell.physiology.org/)

![Fig. 4. Fluorescence resonance energy transfer (FRET) measurements by fluorescence spectrometry. Gastric glands grown in Matrigel-coated dishes were infected with rAd expressing YEC-WT (A and B). YEC-TA (C and D), or YEC-TD (E and F). After infection for 48 h, cultured glands were harvested and homogenized in PBS containing 1 mM EDTA. Lysates were cleared by centrifugation before fluorescence measurements. For each sample (mock infected, YEC-WT, YEC-TA, or YEC-TD), emission spectra were collected from 450 to 550 nm at an excitation wavelength of 425 nm. At this excitation wavelength, CFP is excited, and FRET emission is expected to peak at 526 nm. YFP emission spectra were also collected from 450 to 550 nm at an excitation wavelength of 485 nm. SDS was then added to each sample to obtain a final concentration of 1% SDS, and the same spectra were collected. The conformation of most proteins is affected by 1% SDS, but it leaves green fluorescent protein (GFP) derivatives intact. Data collected from mock-infected glands were used to subtract background/autofluorescence from each sample.](http://ajpcell.physiology.org/)
new information delivered here is the dramatic increase in T567 phosphorylation with any of the gland samples treated with CLA or OKA. The combination of phosphatase inhibitors with histamine plus IBMX did not exert further effects. When probed with anti-ezrin antibody, similar amounts of the total ezrin signal were detected for all samples (Fig. 2C), indicating that the increased T567 phosphorylation was not due to increased expression of total ezrin. From three independent experiments, an average 4.09 ± 0.47 (mean ± SE, \( P < 0.01 \))-fold increase in the level of T567 phosphorylation was observed with CLA-treated glands. The enhanced levels of T567 phosphorylation observed with protein phosphatase inhibitors is consistent with a relatively high turnover at this ezrin phosphorylation site. If we make the generalizing assumption that all ezrin in CLA-treated sample was phosphorylated, we can estimate that, in the steady state, <20% of total ezrin is phosphorylated at T567 in stimulated parietal cells.

Conformational change induced by T567 phosphorylation. The above results suggest that ezrin T567 phosphorylation is a finely regulated event in parietal cell function. It would be interesting, therefore, to know if the conformation of ezrin is changed upon T567 phosphorylation. To probe for this, we made TA and TD mutants on the construct YEC, which has been previously used to show the N-C binding conformation of ezrin by FRET analysis (39). As previously noted, expressed YEC-WT is apparently located randomly throughout the pari-

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**Fig. 5.** FRET measurements by live cell confocal microscopy. Isolated gastric parietal cells infected with rAD expressing YEC-WT (A), YEC-TA (B), or YEC-TD (C) were imaged with a confocal microscope for FRET measurements. YFP signals were collected at 526–537 nm with a 514-nm laser. CFP signals were collected at 462–505 nm with a 458-nm laser. FRET signals were collected at 526–537 nm with a 458-nm laser. After the collection of the initial data, photobleaching was done with 100% laser strength for 20 iterations at 514 nm. The same set of YFP, CFP, and FRET signals were collected after photobleaching. FRET quantitation and normalization were done as described in **MATERIALS AND METHODS**, and mean values of normalized FRET (NFRET) values for YEC-WT (5 samples), YEC-TA (4 samples), and YEC-TD (4 samples) were plotted for comparison (D). Error bars represent SDs. Bars = 10 μm. \( I_{\text{CFP}} \), intensity of CFP.

**Fig. 6.** Enhanced F-actin binding with YEC-TD. Nonmuscle G-actin was reconstituted to 1 mg/ml in low-salt G-actin buffer (buffer A). Rabbit gastric glands expressing YEC-WT, YEC-TA, or YEC-TD were homogenized in buffer A. Lysates were cleared at 1 h at 300,000 g at 4°C before being used. Polymerization was started by mixing the actin monomer solution, the gland lysate, and a 10× salt solution. Control samples with only actin and control samples with only lysate were made simultaneously. Actin-lysate mixtures were incubated at room temperature for 1 h. F-actin and its binding proteins were pelleted by ultracentrifugation. Samples of both supernatant (S) and pellet (P) are shown. A: all cosedimentation and control samples were separated on an SDS-PAGE gel and stained with Coomassie blue. B: immunoblot assay with anti-GFP. C: reprobing of the same blot with anti-ezrin after the blot had been stripped. Longer exposures were shown for B, left, and C, left, than B, right, and C, right, to show weak signals in the pellets of control samples.
eral cell with little or no special site of localization (Fig. 3). The YEC-TA mutant showed similar random localization. However, the TD mutation had a significant effect on the localization of YEC; it was found concentrated around membranes, mainly basolateral membranes and sometimes apical membranes (Fig. 3; see also Fig. 5). Thus, the TD mutation was able to rescue NH₂-terminally tagged ezrin from random localization.

Lysates were made from glands expressing YEC-WT, YEC-TA, and YEC-TD for FRET measurements. Fluorescence emission spectra were recorded both at an excitation 425 nm and excitation of 485 nm (Fig. 4). Excitation at 425 nm excites CFP, whereas excitation at 485 nm excites YFP. If there was an energy transfer from CFP to YFP, as in the case of FRET, excitation at 425 nm would induce an emission peak at 526 nm, the typical YFP emission peak. FRET analysis with YEC-WT was apparent, as shown in Fig. 4A: an emission peak at 526 nm was detected at 425-nm excitation, which disappeared when the same sample was mixed with SDS, indicating that the FRET signal was conformation dependent. Similar results were obtained with YEC-TA, indicating that the TA mutation did not affect the ezrin N-C binding conformation (Fig. 4C). However, the FRET signal was greatly diminished with the TD mutation (Fig. 4E). All of these constructs showed the typical YFP emission spectra when excited at 485 nm (Fig. 4, B, D, and F). Comparing all spectra, the YEC-TD spectrum was different in that, given similar amounts of YFP intensity (a good representation of the protein amount of YEC), it had a much higher CFP intensity, indicating that for the YEC-TD mutant, there was much less energy loss to YFP when CFP was excited.

The constructs described above are suitable for in vivo analysis of the T567 phosphorylation effect on ezrin N-C binding. Gastric parietal cells expressing YEC-WT, its TA mutant, or its TD mutant were subjected to FRET analysis by live cell confocal microscopy. Signals were collected from the CFP channel (CFP excitation and emission), YFP channel (YFP excitation and emission), and FRET channel (CFP excitation and YFP emission). Net FRET signals were calculated by subtraction of the bleed through of CFP and YFP signals from the raw FRET signals. Net FRET signals were then normalized with the respective CFP and YFP signals of each sample. A typical YEC-WT-expressing cell is shown in Fig. 5A, and fluorescent signals were located throughout the cell, as previously documented (39). As expected, FRET was observed on YEC-WT with a normalized FRET value of 0.15 ± 0.03 (mean ± SD, n = 5 cells). Another method to evaluate FRET is to look for an enhancement of the donor signal when the acceptor fluorophore is eliminated by photobleaching. Accordingly, FRET-positive cells were bleached with a 514-nm laser, and the signal from the YFP channel was significantly decreased, which was accompanied by an elevated CFP signal (Fig. 5A). YEC-TA was also distributed throughout the cell, as shown in Fig. 5B, which shows a typical parietal cell expressing YEC-TA. The normalized FRET value was calculated from four cells to be 0.13 ± 0.02. Energy transfer from CFP to YFP was apparent when an enhanced CFP signal was observed after photobleaching of YFP (Fig. 5B). YEC-TD was more localized at the cortical area of the cell than either YEC-WT or YEC-TA (Fig. 5C). Normalized FRET calculated from four YEC-TD cells was 0.02 ± 0.02, significantly lower than that of YEC-WT or YEC-TA (Fig. 5D; P < 0.01). Moreover, bleaching of YFP did not cause an elevation of the CFP signal (Fig. 5C), further evidence that YEC-TD mutant ezrin demonstrated very little FRET between CFP and YFP fluorophores.

Enhanced F-actin binding with T567 phosphorylation. As for other NH₂-terminally tagged ERM constructs (2, 15, 39), YEC-WT and its TA mutant showed random localization throughout parietal cells. Nonetheless, the TD mutant of this construct did show a conformational change of ezrin. In addition, this TD mutant was more localized to membranes than the WT or TA forms of ezrin, indicating that the conformational change may have led to its functional activation. To test this, an
F-actin cosedimentation assay was performed with YEC constructs. YEC-WT, YEC-TA, and YEC-TD expressed in gastric glands were extracted and mixed with β-actin monomers in low-salt G-actin buffer. Salt was then added to the mixtures to initiate actin polymerization. Polymerized actin was sedimented by ultracentrifugation. Figure 6, left, shows control samples; Fig. 6, right, shows samples of YEC-WT, YEC-TA, and YEC-TD plus added β-actin. Coomassie blue staining of the samples separated by SDS-PAGE monitored the location of actin (Fig. 6A). Efficient actin polymerization was observed for all samples with actin added to the mixture. To analyze cosedimented YEC proteins, samples were analyzed by immunoblot analysis with anti-GFP (Fig. 6B), which recognizes CFP and YFP as well as GFP. With control samples containing YEC only, little YEC was detected in the pellet for any of the ezrin constructs, indicating that the lysate alone would not produce a significant yield in the pellet when mixed with salt; thus, the difference among YEC samples could be confidently claimed to be induced by F-actin sedimentation. Indeed, the difference in sedimentation was striking between TD and the WT and TA constructs. With WT and TA, most of the YEC signal remained with the supernatant and only a small fraction was cosedimented with F-actin; whereas with TD, more than half of the YEC signal was cosedimented with F-actin. No significant differences were observed between WT and TA constructs. The same blot was stripped and then reprobed with anti-ezrin, which detected both YEC proteins and endogenous ezrin (Fig. 6C). The YEC signals here were similar to those revealed by anti-GFP. The endogenous ezrin signal was a small surprise in that more endogenous ezrin cosedimented with the YEC-TD sample than with the YEC-WT or YEC-TA samples. Still, in the YEC-TA sample, cosedimentation of ezrin was not as efficient as that of the YEC-TD sample, confirming that the TD mutant binds more efficiently with F-actin than WT ezrin.

**Enhanced membrane association with T567 phosphorylation.** The conformational change induced by T567 phosphorylation also changes the exposure of the NH2 terminal membrane binding domain. To test if this leads to stronger membrane binding of ezrin, a sequential extraction procedure was applied to glands expressing YEC constructs. Glands expressing YEC were extracted first with low-salt buffer A to extract cytosolic ezrin, with high-salt buffer C to extract ezrin loosely bound to the membrane, and finally with 1% Triton X-100 in buffer C to extract ezrin tightly bound to the membrane. Analysis of the various extracted samples by Western blot assay with anti-GFP and anti-ezrin is shown in Fig. 7A. Consistent with the confocal microscopic observations that YEC proteins are not localized as well as native ezrin or ezrin-CFP proteins in general, the majority of the YEC proteins came out in the low-salt buffer extractions, regardless of amino acid residue 567. Although the amount of YEC proteins in the Triton X-100 extractions represent ~10% of total YEC protein, there was an obvious increase in the amount of YEC extracted by Triton X-100 in the TD sample compared with the WT and TA samples. Results of three separate extraction experiments were quantified by directly measuring the YFP fluorescence of each extract and calculating the percentage distribution of the three YEC constructs. Of particular interest was the fluorescence percentages found in Triton X-100 extracts: 6.8 ± 1.5% for YEC-WT, 6.3 ± 3.4% for YEC-TA, and 15.3 ± 3.0% for YEC-TD (means ± SE; Fig. 7B). In every experiment, YEC-TD was more heavily and significantly distributed toward the Triton X-100 extraction than either the YEC-WT or YEC-TA constructs (P < 0.05).

**Tight membrane association of T567-phosphorylated endogenous ezrin.** If the results for YEC-TD binding could be translated to endogenous ezrin, T567 phosphorylation should lead to more membrane binding of native ezrin. Thus resting, stimulated, and CLA-treated glands were sequentially extracted with PBS and 1% Triton X-100 in PBS. Extractions were analyzed by immunoblot assay with anti-T567P and anti-ezrin. In resting and stimulated samples, more of the T567-phosphorylated ezrin was located in Triton X-100 extractions than in PBS extractions (Fig. 8, A and C), whereas most of the total ezrin was extracted with PBS (Fig. 8, B and D), as previously reported (14). After being normalized against the ezrin signal, a small increase (23.5%) of T567 phosphorylated ezrin signal was associated with the stimulated sample, similar to the results shown in Figs. 1 and 2. However, a striking difference was observed between the CLA-treated sample and other samples. Consistent with previous observation (Fig. 2), there was a large increase of T567 phosphorylation.

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**Figure 8.** Tight membrane association of endogenous ezrin phosphorylated at T567. Resting (CIM), stimulated (H/I), and CLA-treated gastric glands were sequentially extracted with PBS containing 1 mM EDTA (PBS) and 1% Triton X-100 in PBS containing 1 mM EDTA (T). Equal fractions of each extraction from all samples were analyzed by immunoblot assay with anti-T567P to reveal T567 phosphorylated ezrin (A). The blot was reprobed with anti-ezrin after being stripped to reveal the total ezrin level (B). Densitometric analyses of band intensities are shown in C for T567P and in D for ezrin.
tion for the CLA sample over the cimetidine sample (Fig. 8, A and C), which led to an overwhelming shift of total ezrin from the PBS extraction to Triton X-100 extraction (Fig. 8, B and D).

Membrane dynamics of ezrin. The above results provide in vitro evidence that T567-phosphorylated ezrin displayed relatively tighter binding to F-actin and membrane than non-phosphorylated ezrin. To evaluate the same question in vivo, FRAP analysis was performed using ezrin-CFP constructs, which behave more like endogenous ezrin than double-tagged YEC protein (38). Portions of parietal cells expressing ezrin-CFP were photobleached with a 458-nm laser, and time series of images were taken before and after bleaching. \( t_{1/2} \) values were calculated as described in MATERIALS AND METHODS. WT ezrin-CFP was expressed mainly on apical membrane vacuoles and to only a slight extent on the basolateral membrane. A typical sample of WT ezrin-CFP is shown in Fig. 9A, with fast fluorescence recovery for ezrin-CFP located on either the apical or basolateral membrane; \( t_{1/2} \) values for apical (15 cells) and basolateral (6 cells) fluorescence were calculated to be 34 ± 11 and 39 ± 18 s (mean ± SD), respectively. The TA mutant of ezrin-CFP showed a similar localization and turnover dynamics as WT. A typical ezrin-CFP-TA sample is shown in Fig. 9B, again with relatively fast fluorescence recovery for both apical and basolateral ezrin-CFP. Mean \( t_{1/2} \) values for apical (11 cells) and basolateral (8 cells) fluorescence were calculated to be 27 ± 17 and 22 ± 8 s, respectively. The TD mutant of ezrin-CFP was distributed differently than the other two constructs, more predominantly being localized to basolateral membranes and cell surface projections like filopodia and lamellipodia. As shown in Fig. 9C, fluorescence recovery for the TD mutant was much slower than the WT or the TA mutant. The \( t_{1/2} \) value for the apical membrane was 106 ± 62 s, significantly longer than that of the WT or the TA mutant (\( P < 0.01 \)). The \( t_{1/2} \) value of the TD mutant at the basolateral membrane (20 cells) was 105 ± 44 s, also significantly longer than that of the WT or the TA mutant (\( P < 0.01 \)). When filopodia were bleached, their fluorescence often failed to come to full recovery. The calculated \( t_{1/2} \) value (6 cells) was 168 ± 75 s, significantly longer than any other
sample (P < 0.02 compared with the basolaterally localized TD mutant).

**DISCUSSION**

**Phosphorylation of T567 activates ezrin.** Evidence gathered in vitro (10) and in vivo (39) supports the concept that dormant ezrin takes on a conformation in which the NH$_2$ terminal binds to the COOH terminal. Here, FRET measurements on live cells provided in vivo evidence (Fig. 5) that ezrin T567 phosphorylation promotes the dissociation of N-C binding and extends in vitro evidence from previous studies (17, 22). The conformational change of ezrin is a “switch” for its activity: opening up of the ezrin N-C binding directly leads to increased affinity to F-actin (Fig. 6) and membranes (Figs. 7 and 8). The increased binding affinity influenced the localization of YEC proteins in confocal microscopy; the YEC-TD mutant with an opened conformation was more localized on membrane/cytoskeleton structures than the WT or the TA mutant (Fig. 3). This observation was further confirmed by FRAP analysis with these same YEC constructs (data not shown): fluorescence recovery of the YEC-TD mutant typically took hundreds of seconds, while the recovery of the WT or the TA mutant was instantaneous, indicating cytosolic localization. Based on these facts, it is not surprising that the expression of the ezrin TD mutant leads to the formation of more surface membrane structures (8, 12).

**The dormant N-C conformer of ezrin binds to membrane sites.** Present data also demonstrated that in its dormant N-C conformation, the NH$_2$ terminal (membrane binding domain) is more important in membrane surface localization than the COOH terminal (F-actin binding domain). This is apparent by the diffuse localization of ezrin that occurs when the NH$_2$ terminus is blocked with a YFP tag in the case of WT and TA mutant ezrin (Fig. 3, A and B; also see Refs. 2 and 39), in contrast to normal membrane localization when the fluorescent tag is at the COOH terminal (Fig. 9, A and B). While dormant ezrin certainly binds to membrane loci, activation by phosphorylation or TD mutation leads to apparent enhanced membrane/actin association (Figs. 7 and 8).

**High turnover of T567 phosphorylation is necessary for functionally dynamic ezrin.** Since ezrin is an important component of the parietal cell apical membrane, where complex membrane structures are necessary for the recruitment and turnover of large amounts of H$^+$-K$^+$-ATPase, it was expected that ezrin activation by T567 phosphorylation would facilitate the formation and maintenance of apical membrane/cytoskeleton structures, thus allowing more efficient acid secretion. However, an early study (31) in parietal cells suggested a low amount of $^{32}$P incorporation into threonine on ezrin. We now suspect that the reason for not detecting T567 phosphorylation was due to the low-level phosphorylation at this site and possible high level of phosphatase activity in the parietal cell. The following evidence reported here supports the notion that high turnover of ezrin T567 phosphorylation is part of the mechanism for ezrin activity regulation: 1) T567 phosphorylation increased in response to stimulation of the parietal cell via the histamine receptor/cAMP-mediated pathway (Fig. 1); 2) the steady-state level of T567 phosphorylation is relatively low (Figs. 2B and 8); and 3) accumulation of T567 phosphorylation is fast when protein phosphatase is inhibited (Fig. 2B).

One noteworthy indirect point of evidence for the high turnover of T567 phosphorylation is that no activity (F-actin or membrane binding) differences were observed between the ezrin TA mutant and WT.

Regulation of phosphorylation/dephosphorylation is likely a common theme for the functional activity of ezrin. In T-cell receptor-stimulated Jurkat T cells, an increase of tyrosine phosphorylation of ezrin was not observed until phosphatase inhibitors were applied (9), indicating a low level of tyrosine phosphorylation, but high turnover, in the steady state. This mechanism may extend to other members of ERM proteins. In neutrophils, phosphorylation of moesin on T558 was detected, which has to be constantly dephosphorylated for the cell to move to its target (36). When T558 dephosphorylation was inhibited by CLA, T558 phosphorylation accumulated, which was correlated with the impairment of release and retraction of the posterior foot (uropod) of a moving cell.

Different from the negative effect in neutrophils, inhibition of phosphatase with CLA in parietal cells stimulated acid secretion. The greatly increased level of ezrin T567 phosphorylation (Fig. 2) observed with CLA seems to be contrary to the fact that introduction of the ezrin TD mutant into parietal cells did not lead to enhanced acid secretion (38). In fact, TD mutant expression is very different from CLA treatment. One important difference is that CLA treatment clearly influences a multitude of phosphorylation events (32), several of which could have profound effects on secretory activation. A second difference is that TD mutant expression required a relatively short-term treatment, lasting 48–72 h, whereas CLA treatment was a short-term (25 min) treatment. If a protein phosphatase inhibitor was so specific as to inhibit only the dephosphorylation of ezrin T567, then long-term effects of such an inhibitor might lead to the same result as expression of the TD mutant in parietal cells. A third difference is that the TD mutant mimics the permanent phosphorylation only at T567, but CLA may affect the phosphorylation level at more than one site in ezrin. In parietal cells, ezrin was first shown to be phosphorylated at serine, and two-dimensional gel analysis suggested multiple phosphorylation sites on ezrin (31). Moreover, a mutation study (37) demonstrated that phosphorylation on

**Fig. 10.** Scheme for dynamic membrane-cytoskeleton interactions promoted by turnover of phosphorylation on ezrin T567 and consequent accessibility of actin binding sites by conformational change. N, NH$_2$ terminal; C, COOH terminal. The NH$_2$ terminal membrane binding site on ezrin is permanently associated with the plasma membrane through adaptor proteins (shaded ovals). Phosphorylation of ezrin T567 leads to conformational change, disruption of the NH$_2$ terminal-to-COOH terminal interaction, and exposure of the COOH terminal F-actin binding site. Dephosphorylation restores the NH$_2$ terminal-to-COOH terminal binding conformation and relaxes the membrane-actin interaction. Thus, phosphorylation turnover serves to make and break the actin association events and apply lateral tension force to reposition the newly recruited membrane over the actin cytoskeleton.
Ser<sup>56</sup> may regulate ezrin activity. It is intriguing whether these phosphorylation events are subjected to different temporal and spatial regulation.

The active ezrin conformation binds avidly to F-actin. Since ezrin-CFP showed a closer localization to endogenous ezrin than YEC constructs, FRAP analysis with ezrin-CFP constructs should provide better representation of the dynamics of endogenous ezrin. The fluorescence recovery time for the ezrin-CFP TD mutant after photobleaching was significantly longer than that for the WT or the TA mutant (Fig. 9). The delay of the exchange among different ezrin-CFP TD molecules suggests that this form is more firmly locked in complex with F-actin, or the membrane, or both. On the other hand, the activity of interacting partners (e.g., F-actin or membranes) might be restricted by this sticky TD ezrin mutant. Thus, the TD mutant could alter normal membrane activity directly or through F-actin. This “fixation” effect could happen on both apical and basolateral membranes. Since there is an abundant supply of native ezrin in the cell with full phosphate turnover potential, the net binding affinity of the newly expressed TD mutant might be favored at the basolateral surface. Fixation of basolateral membrane is not a major problem for the membrane itself, but TD mutant ezrin is trapped there. The accumulation of the ezrin TD mutant over time may finally promote the formation of cell surface structures, like lamellipodia, filopodia, membrane ruffles, and tufts of microvilli, accumulating the actin and membrane pools from other regions of the cell (38).

Based on the present data, we now propose that ezrin activity is precisely regulated by T567 phosphorylation and dephosphorylation. Both events are rapid, but in the steady state, dephosphorylation predominates, so that at any given time, the majority of ezrin molecules are not phosphorylated (at least in the parietal cell). Once a specific kinase has phosphorylated T567, the ezrin molecule is activated, tightly linking the membrane to the cytoskeleton. Then, ezrin itself becomes the substrate of phosphatase, being released from F-actin and going back to its dormant state and the start of a new cycle. The importance of the cycle of ezrin (open → close) is to keep all the membrane activity in a dynamic state, not fixed, as demonstrated by the FRAP data.

Conclusions and perspective. A bulky NH<sub>2</sub> terminal fluorescent tag blocks the membrane binding capacity (i.e., localization) for WT or TA mutant constructs of ezrin (Figs. 3; see also Refs. 2 and 39)). On the other hand, TD mutant ezrin expressed with NH<sub>2</sub> terminal fluorescent protein is bound to basolateral and apical membranes in parietal cells (Fig. 3). The FRET data signify that WT and TA mutant ezrin are in the N-C conformer state, whereas the TD mutant is more in the open conformer state (Figs. 4 and 5). Thus, the binding of the YEC-TD mutant is due to exposure of the actin binding domain in the open state. This is supported by the enhanced F-actin binding capacity of the TD mutant compared with the WT or the TA mutant (Fig. 6).

With the fluorescent tag positioned only at the COOH terminal (e.g., ezrin-CFP), all three ezrin constructs (WT, TA, and TD) are localized to membrane surfaces. WT and TA are primarily localized to the apical membrane (Fig. 9, A and B), and, since these constructs are likely to be in the dormant state, ezrin in the N-C conformation still binds to the membrane. TD initially binds to both apical and basolateral membranes, but, with time, becomes more prominently associated with the basolateral surface (data not shown). Molecular interactions from the FRAP data suggest that TD binding to apical or basolateral membranes is much tighter than binding of TA or WT (Fig. 9, A–C), most likely accounted for by the exposed actin binding domain of the open conformation.

At the steady state in the nonsecreting parietal cell, only ~20% of total endogenous ezrin is phosphorylated at T567 (Figs. 2 and 8), yet all of the endogenous ezrin is associated with the membrane [mostly apical (14)] and there appears to be high turnover of phosphate at T567 that favors the dephosphorylated form. These data are again consistent with an effective membrane binding site for ezrin in the N-C conformation. The high turnover of T567 phosphorylation suggests cyclic and variable F-actin binding capability of ezrin via regulated protein kinase and phosphatase activities.

On the basis of these correlations, we make the following conclusions regarding the state and cellular activities of ezrin. First, WT and TA are in the N-C conformer state, and TD is in the open conformation. Second, ezrin in the dormant N-C conformation has NH<sub>2</sub> terminal membrane binding sites. Third, ezrin in the open conformation has both NH<sub>2</sub> terminal and F-actin binding sites. Fourth, the open conformation is much stickier than the closed conformation. Fifth, there is a rapid turnover of T567P with the steady state favoring the dephosphorylated form. Finally, ezrin and its phosphorylation regulatory complex has properties of a motor protein, entailing 1) a potentially “permanently attached” membrane binding site, 2) a site of phosphate turnover that involves a conformational change, 3) an F-actin binding site that changes affinity depending on phosphorylation.

We suggest that the purpose of the ezrin motor complex is to maintain a dynamic relationship between the membrane and supporting cytoskeleton in the face of varying membrane surface areas and filamentous actin lengths that would occur with changes in membrane recruitment and surface extensions. Figure 10 shows a schematic representation of the functional consequence of T567 phosphate turnover in ezrin.

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C884 HIGH Turnover of Ezrin T567 Phosphorylation